EUKARYOTIC CELL, Sept. 2006, p. 1571–1576 Vol. 5, No. 9
1535-9778/06/$08.00+0 doi:10.1128/EC.00100-06

Symbiotic "Archaezoa" of the Primitive Termite *Mastotermes darwiniensis* Still Play a Role in Cellulase Production

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Received 8 April 2006/Accepted 6 July 2006

The relictual *Mastotermes darwiniensis* is one of the world's most destructive termites. Like all phylogenetically basal termites, it possesses protozoa in its hindgut, which are believed to help it digest wood. L. Li, J. Frohlich, P. Pfeiffer, and H. Konig (Eukaryot. Cell 2:1091–1098, 2003) recently cloned the genes encoding cellulases from the protozoa of *M. darwiniensis*; however, they claimed that these genes are essentially inactive, not contributing significantly to cellulose digestion. Instead, they suggested that the protozoa sequester enzymes produced by the termite in its salivary glands and use these to degrade cellulose in the hindgut. We tested this idea by performing gel filtration of enzymes in extracts of the hindgut, as well as in a combination of the salivary glands, foregut, and midgut. Three major cellulases were found in the hindgut, each of which had a larger molecular size than termite-derived salivary gland enzymes. N-terminal amino acid sequencing of one of the hindgut-derived enzymes showed that it was identical to the putative amino acid sequence of one mRNA sequence isolated by Li et al. (Eukaryot. Cell 2:1091–1098, 2003). The overall activity of the hindgut cellulases was found to be of approximately equal magnitude to the termite-derived cellulases detected in the mixture of salivary gland, foregut, and midguts. Based on these results, we conclude that, contrary to Li et al. (Eukaryot. Cell 2:1091–1098, 2003), the hindgut protozoan fauna of *M. darwiniensis* actively produce cellulases, which play an important role in cellulose digestion of the host termite.

Terrestrial ecosystems annually fix (6.0 to 6.3) $\times$ 10^{16} g of carbon as plant biomass (17). Three major constituents of such biomass are cellulose, hemicellulose, and lignin. Decomposition/mineralization of these materials is carried out by not only bacteria and fungi but also soil macro-invertebrates such as termites (Insecta: Isoptera) (29). Termites, with their associated microbial symbionts, effectively dissimilate a significant proportion of the cellulose, as well as the hemicellulose, that they ingest (1).

So-called lower termites, distinguished from "higher" termites (family Termitidae) by the presence of abundant parabasilid and oxymonad protozoa in the hindguts, have a unique cellulose digestion system, which is supported by cellulases of both termite and protozoan origins (13, 22). Advances in molecular biological techniques have enabled the characterization of both termite- and protozoa-derived cellulases at the amino acid and cDNA sequence levels (28). Termite-derived cellulases belong to glycosyl hydrolase family 9 (GHF9), while those from protozoa (so far characterized from the termite species *Coptotermes formosanus* and *Reticulitermes speratus*) belong to GHFs 5, 7, and 45 (4, 5, 12, 16, 26). It should be noted that GHFs 5, 7, 9, and 45 do not share any sequence similarity and are thus assumed to have evolved independently.

Mastotermes darwiniensis is known to be the most primitive species of termite (8). In the 1980s, both termite endogenous and protozoan cellulases were shown to function in the gut of *M. darwiniensis* on the basis of cellulase activities detected individually from the midguts (termite origin) and hindguts (protozoa origin) by gel filtration chromatography (25). Recently, Li et al. (9) attempted to purify the protozoa-derived cellulase of *M. darwiniensis* using ion exchange chromatography. (The term "Archaezoa" was used by Li et al. [9] to describe the cellulolytic symbionts; however, this term is no longer considered valid to refer those single-celled eukaryotes that lack mitochondria [2, 18].) Surprisingly, the N-terminal amino acid sequence of the cellulase purified from the hindgut extract was identical to that of a cellulase from salivary glands of the host termites (GHF9) (9). From the hindgut protozoan fauna, Li and colleagues also found mRNAs that encode cellulases of GHF45, similar to those of protozoa from other termites. However, the corresponding proteins were not isolated during chromatography of the protozoan extract. Consequently, Li and colleagues concluded that the GHF45 cellulases do not play a significant role in cellulose digestion in the hindguts; instead, they suggested that termite GHF9 cellulases from the salivary glands were acquired and utilized by the symbiotic protozoa in the hindguts.

Since ion exchange chromatography can preferentially purify proteins within a narrow range of pI, it may not be suitable for analysis of a wide spectrum of proteins in the hindgut of termites. Thus, it is possible that Li et al. (9) overlooked the presence of protozoan cellulases. To test this possibility, we attempted to fractionate the hindgut cellulases by gel filtration chromatography, which separates proteins based on molecular sizes. Here, we report the purification and N-terminal sequence of a GHF45 protozoan cellulase and discuss its contribution to cellulose digestion in *M. darwiniensis*.

(A. Takase participated in the present research under the

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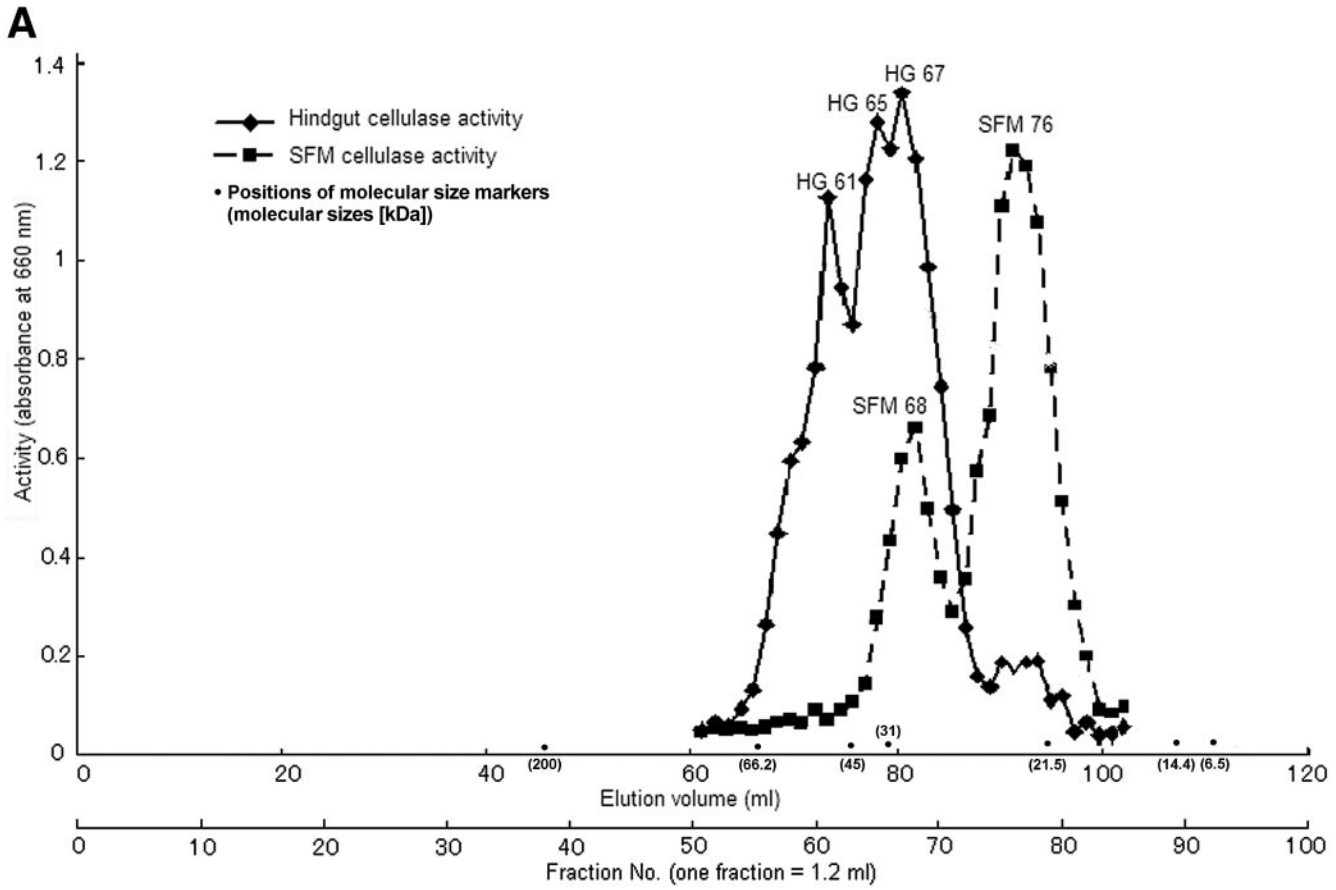

FIG. 1. Purification of cellulases from *M. darwiniensis*. (A) Elution profiles of the extracts from the SFM mixture and from the hindgut through a HiLoad 16/60 Superdex 75 prep grade gel filtration column. The solid line with diamonds and the broken line with squares indicate cellulase activities on CMC of the hindgut and SFM extracts, respectively. One fraction is 1.2 ml. Sodium acetate buffer (0.1 M; pH 5.5) was used for elution. Elution positions of molecular size standards are designated with filled circles above the horizontal axis with their molecular sizes (kDa). Proteins used as molecular size standards are described in the text. (B) Activity staining of concentrated cellulase peaks (derived from Superdex gel filtration described in panel A) run on a native PAGE gel (0.1% CMC used as a substrate).

guidance of H. Watanabe and G. Tokuda in partial fulfillment of the requirements for a Master's degree from the University of Ryukyus, Nishihara-cho, Okinawa, Japan.)

MATERIALS AND METHODS

Termites. *M. darwiniensis* was collected at road reserves of Buffalo Creek Road, Casuarina, Darwin, NT, Australia, on 5 December 2006. Living termite specimens were transferred to the University of Sydney, and salivary glands, foreguts, and midguts (SFM) were excised from a total of 30 pseudergates (workers) and stored in solution U (24) at −30°C. Hindguts were also excised from the termite individuals and stored in the same solution at −30°C.

Crude extract preparation and gel filtration. The stored tissues (the hindguts or the SFM mixture) were homogenized in 500 μl of 10× complete mini ETDA-free protein inhibitor cocktail solution (Roche Diagnostics GmbH, Mannheim, Germany). The homogenate was centrifuged at 20,400 × *g* for 5 min, and then the supernatant was filtered using a spin column membrane filter with a 0.22-μm pore size (SUPREC-01; Takara Bio Inc., Otsu, Shiga, Japan). Henceforth, the filtrate is referred to as the "crude extract". The crude extract was applied to a gel filtration column (HiLoad 16/60 Superdex 75 prep grade; GE-Healthcare Bio-Science, New Jersey). Proteins were eluted with 0.1 M sodium acetate buffer (pH 5.5) at a flow rate of 1 ml/min. Fractions of 1.2 ml were collected up to the 100th fraction.

Native and SDS-PAGE. Active fractions from the gel filtration were accumulated and concentrated by ultrafiltration (cutoff molecular weight, 10,000) (Ultrafree-MC; Millipore, Massachusetts), and one-half volume of native sample buffer (161-0738; Bio-Rad, California) or Laemmli's sample buffer (161-0737; Bio-Rad) with 5% (vol/vol) 2-mercaptoethanol was added. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the sample was heated at 100°C for 5 min after the addition of Laemmli's sample buffer. A precast polyacrylamide gradient gel (5 to 20%) (e-PAGEL; item E-R520L; ATTO Corporation, Tokyo, Japan) was employed for PAGE under either native conditions (native PAGE) or denatured conditions (SDS-PAGE). The electrophoresis was conducted in Tris-glycine buffer (161-0734; Bio-Rad Laboratories, Hercules, CA), diluted with pure water for native PAGE and 0.1% (wt/vol) SDS for SDS-PAGE, at a constant current of 10 mA for the first 30 min then at 20 mA for 90 min with a PAGERUN electrophoresis apparatus (ATTO Corp.). Activity staining was performed on a native PAGE gel (10%) containing 0.1% carboxymethyl cellulose (CMC) as described previously (13).

Proteins undergoing native PAGE were analyzed further as follows. Of four lanes loaded with the same concentrated peak from the gel filtration, one lane was used for Coomassie brilliant blue R-250 (161-0436; Bio-Rad Laboratories) staining, two lanes were used for transblotting, and the remaining lane was used for fractionation. Here, the lane was sectioned into small pieces with a width of 2.1 mm using a gel slicer (skewered razor blades with two long screw bolts spaced with washer rings). Each sectioned piece was put into 150 μl of pure water in a 1.5-ml tube, and the tubes were shaken at 1,000 strokes per min for 2 h at room temperature. After the shaking step, the water solution in each tube was recovered and used for enzymatic measurements.

Measurements of molecular masses. Molecular sizes of the active fractions were measured via gel filtration and SDS-PAGE. The gel filtration chromatography was calibrated with molecular mass markers (myosin, 200 kDa; β-galac-

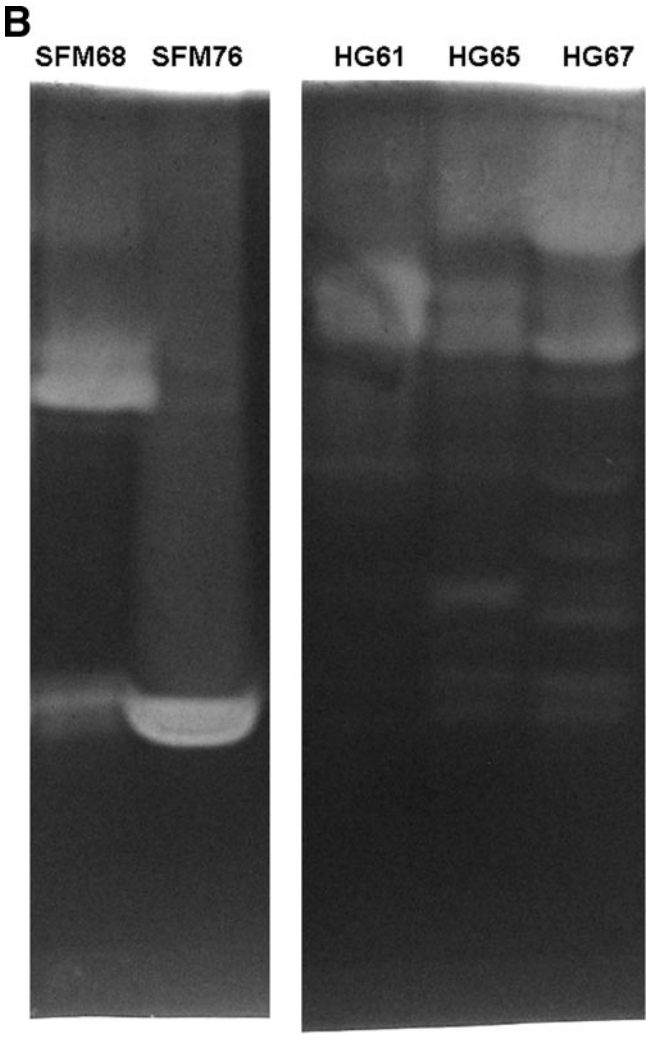

FIG. 1—*Continued.*

tosidase, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa; aprotinin, and 6.5 kDa [item 29458-24; Nakalai Tesque, Kyoto, Japan]). In gel filtration, the molecular masses of the samples were calculated by fitting elution volumes to fitting curves. The molecular masses on SDS-PAGE were estimated by comparing sample bands with recombinant ladder markers (10 to 250 kDa; XL-Ladder Broad; APRO Life Science Institute, Inc., Tokushima, Japan).

Measurements of cellulase activity. An aliquot of enzymatic sample (20 μl) from the gel filtration was added to 200 μl of 1% (wt/vol) sodium CMC solution in sodium acetate buffer (0.1 M; pH 5.5) at 37°C for 30 min. After incubation, 2 ml of tetrazolium blue reagent (7) was added, and the mixtures were heated in boiling water for 5 min. All reactions were done in glass test tubes (12 by 105 mm). Generated reducing sugars from CMC were measured photometrically at 660 nm. For fractions from the native PAGE, 25 μl of an enzyme sample was added to 100 μl of the substrate solution and incubated for 1 h at 37°C. To measure reducing sugars, 200 μl of tetrazolium blue reagent was added and heated for 5 min at 95°C using a heating block. All reactions were conducted in 1.5-ml disposable tubes. Absorbance at time zero was used as blank.

Transblotting of proteins in PAGE gel. Proteins in the PAGE gel were transferred to a polyvinylidene difluoride (PVDF) membrane (Sequi-Blot, item 162-0182; Bio-Rad) using a semidry transfer cell (TRANS-BLOT SD; Bio-Rad) with transfer buffer (49 mM Tris, 39 mM glycine, and 0.0375% [wt/vol] SDS; pH not adjusted) soaked in extra-thick blotting paper (Bio-Rad Laboratories) at 15V for 90 min. Proteins on the membrane were stained with Coomassie brilliant blue R-250 and destained with 40% methanol solution.

N-terminal amino acid sequencing. Protein bands on the PVDF membrane corresponding to relevant positions in SDS-PAGE gels or active positions in the native PAGE gel were cut out and treated with iodoacetamide to break disulfide bonds in the proteins. The treated membrane pieces were applied to a protein sequencer (Hewlett Packard G1005A or Applied Biosystems Procise 494 cLC, depending on required sensitivity) to analyze N-terminal amino acid sequences. The iodoacetamide treatment and following analytical processes were commissioned to the APRO Life Science Institute, Inc., Japan.

RESULTS AND DISCUSSION

Gel filtration chromatography and activity staining. Fig. 1A shows elution profiles of cellulolytic activities in the SFM and hindgut extracts of *M. darwiniensis*. The gel filtration of the SFM extract, which is expected to contain endogenous cellulases of the termite, showed two major peaks (fraction no. SFM 68 and 76) of cellulase activity. On the other hand, the hindgut (HG) extract, which is expected to contain protozoan cellulases and/or endogenous cellulases, consisted of three major peaks (fractions HG 61, 65, and 67) and fractions with trace activities (HG 75 to 80). These elution profiles suggest that two of the major cellulases (eluted in fractions HG 61 and 65) of the hindgut extract are larger than those eluted in the two major peaks of the SFM extract, although one of the major cellulases (eluted in the fraction HG 67) of the hindgut extract has a molecular size similar (but not identical) to one of the major cellulases in the SFM extract (eluted in fraction SFM 68). The minor active fractions (HG 75 to 80) of the hindgut extract overlapped with the other major peak (SFM 76) of the SFM extract.

After concentration of cellulase peaks from the gel filtration (SFM 68 and 76 and HG 61, 65, and 67), an aliquot (10 μl) of each concentration was applied for activity staining by native PAGE (Fig. 1B). For this experiment, the HG 67 peak was concentrated without its shoulders (HG 66 and 68). The result showed that the hindgut cellulases (HG 61, 65, and 67) did not include enzymes in common with those of the SFM mixture (SFM 68 and 76). Thus, the results supported an independent origin of the hindgut cellulases from those of the salivary glands, foregut, and midgut.

Identification of hindgut cellulases. To identify further the cellulases present in the hindgut, HG 67 and its shoulder fractions (HG 66 and 68) were pooled and subjected to native PAGE. After the electrophoresis, a lane of the PAGE gel was sectioned into 29 equal pieces that were subsequently numbered (Fig. 2). Cellulolytic activities were recovered from the gel pieces at positions 5 (HG 67-5) and 14 (HG 67-14) (Fig. 2A). This result suggests that the fraction HG 67 (as well as shoulder fractions) contained two types of cellulase, which differed in size. Since it is possible that HG 67 contained the same cellulase that is primarily eluted in fraction HG 65, proteins in fraction HG 65 were also analyzed by native PAGE. The band pattern of the fraction HG 65 (Fig. 2C) was almost the same as that of HG 67 (Fig. 2B); however, only a trace of cellulase activity was recovered from the gel piece corresponding to position HG 67-5 (result not shown). Thus, it appears that HG 65 does not contain a significant amount of the HG 67-5 cellulase.

The active bands (corresponding to the HG 67-5 and HG 67-14) were blotted onto a PVDF membrane and then excised and analyzed with a protein sequencer. N-terminal amino acid

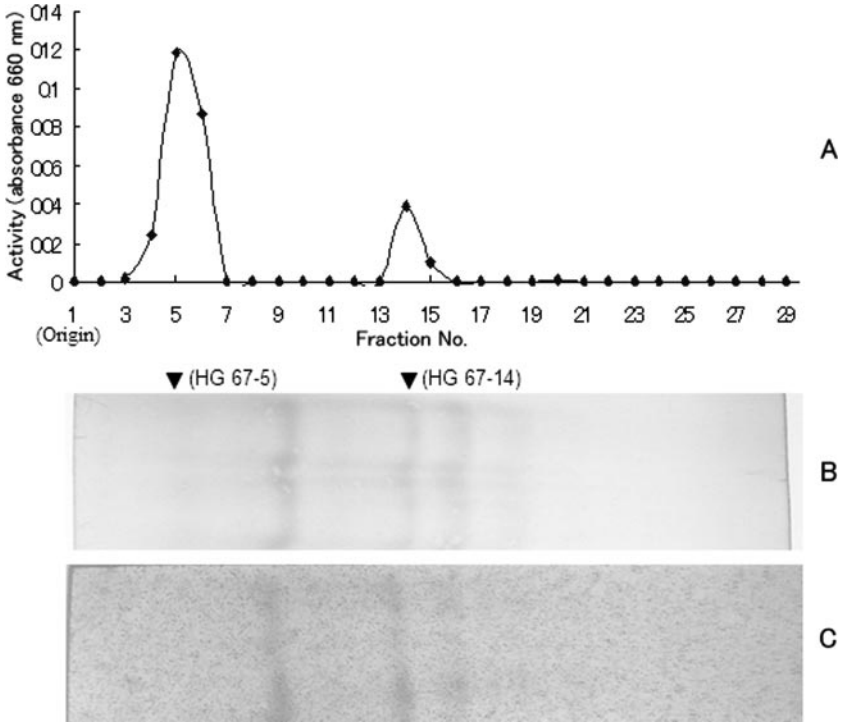

FIG. 2. Native PAGE for gel filtration fractions of the hindgut extracts. (A) Eluted cellulase activity from gel pieces of native PAGE (5 to 20% gel) of the gel filtration fractions HG 66 and HG 67. (B and C) Transblotted image of native PAGE (HG 67 in panel B and HG 65 in panel C). Arrowheads indicate samples which were cut for N-terminal sequencing (HG 67-5 and HG 67-14). Conditions of native PAGE and transblotting are described in the text.

TABLE 1. Comparison of the N-terminal amino acid sequences of the endogenous and hindgut cellulases from amino acid sequencing and predicted amino acid sequences from cDNA

Actual enzyme or accession no. of predicted enzyme[a]	Origin	Amino acid sequence[b]	Molecular mass (value without signal peptides [kDa])
HG cellulase			
HG 67-5	*M. darwiniensis* hindgut	----------**KDYSGNGQTT**	<30
CAD39197	*M. darwiniensis* hindgut protozoa	*MIVVFVIGALC***KDYSGNGQTT***RYWDCCKPSCSWSKKAQVS*	23.3 (22.2)
CAD39199	*M. darwiniensis* hindgut protozoa	*MFVAFVIGALC***KDYSGNGQTT***RYWDCCKPSCSWSKKAQVS*	23.3 (22.2)
CAD39200	*M. darwiniensis* hindgut protozoa	MFVAFVIGALC**KD**F**SGNGQTT**RYWDCCKPSCSWSKKAQVS	23.3
CAD39198	*M. darwiniensis* hindgut protozoa	MIVVFVIGALC**KDYSG**S**G**K**TT**RYWDCCKPSCSWSKKAQVS	23.3
HG 67-14	*M. darwiniensis* hindgut	AIKVADECIP (IKVADECIPL)	<30
SFM cellulase			
SFM 76-50K	*M. darwiniensis* salivary glands, foregut, and midgut	----------**AYDYN** ----------**(AYDYK)**	50
CAD54726	*M. darwiniensis* salivary glands (Cel1)	MRVLLCLLSAFALCQG**AYDYN**DVLTKSLLFYEAQRSGKLP	49.2 (47.5)
CAD54727	*M. darwiniensis* salivary glands (Cel2)	MRVLLCLLSAFALCQG**AYDYK**DVLTKSLLFYEAQRSGKLP	49.5 (47.8)
CAD54728	*M. darwiniensis* salivary glands (Cel3)	MRVLLCLLSAFALCQG**AYDYK**DVLTKSLLFYEAQRSGKLP	49.2 (47.4)
CAD54729	*M. darwiniensis* salivary glands (Cel4)	MRVLLCLLSAFALCQG**AYDYK**DVLTKSLLFYEAQRSGKLP	49.0 (47.3)
CAD54730	*M. darwiniensis* salivary glands (Cel5)	MRVLLCLLSAFALCQG**AYDYN**DVLTKSLLFYEAQRSGKLP	49.0 (47.3)
BAA33708	*Nasultitermes takasagoensis* midgut (NtEG)	MRVFLCLLSALALCQA**AYDYK**QVLRDSLLFYEAQRSGRLP	49.4 (47.6)
BAA31326	*Reticulitermes speratus* salivary glands (RsEG)	MKVFVCLLSALALCQA**AYDYK**TVLSNSLLFYEAQRSGKLP	48.6 (46.9)
BAA34050	*Reticulitermes speratus* salivary glands (RsEG2)	MKVFVCLLSALALCQA**AYDYK**TVLSNSLLFYEAQRSGKLP	48.6 (47.0)

[a] Accession numbers are for the GenBank protein database. HG 67-5 and HG 67-14 are fractions 5 and 14, respectively, of the native PAGE from fraction 67 of the gel filtration of the hindgut extract. SFM 67 is fraction 67 from the gel filtration of the SFM extract. Positions of the fractions from the gel filtration are described in the legend of Fig. 1. Detailed conditions of each extract and the gel filtration are described in the text.

[b] The amino acid sequences designated as signal peptides in the GenBank database are underlined. The amino acid sequences predicted as signal peptides by comparison with the N-terminal amino acid sequence of HG 67-5 are italicized. A second sequence from a minor component in the sample is shown in parentheses.

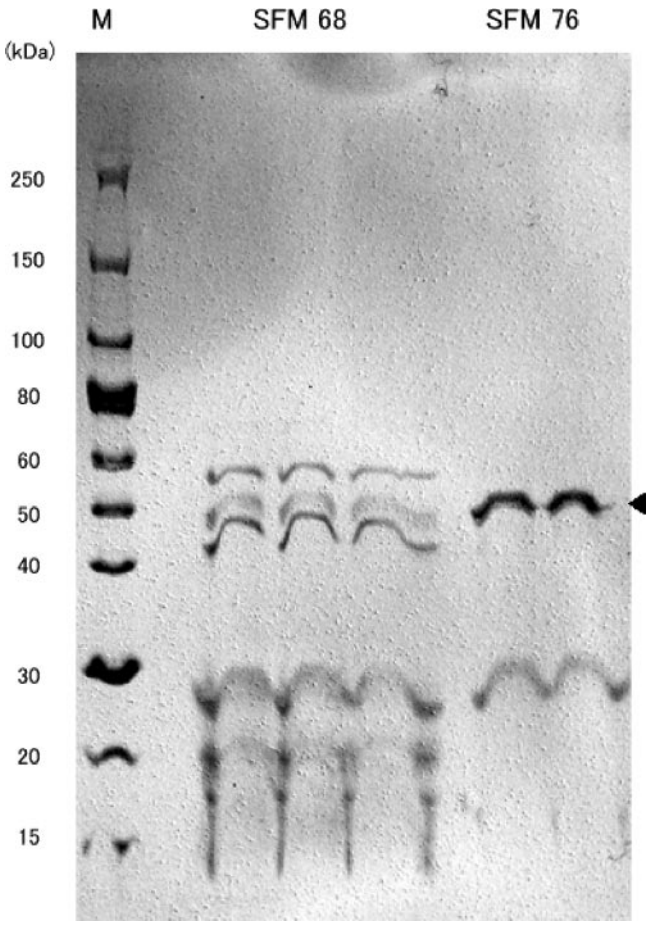

FIG. 3. SDS-PAGE electrophoretograms of SFM 68 and SFM 76 on a PVDF membrane. Fractions containing cellulase activity (SFM 68 and SFM 76) were concentrated and separated by SDS-PAGE. Proteins in the gel were transblotted onto a PVDF membrane and stained with Coomassie brilliant blue R-250. Lane M was run with recombinant ladder markers. The filled arrowhead indicates cutout bands for N-terminal protein sequencing. Detailed conditions for the gel filtration, SDS-PAGE, and the transblotting are described in the text.

sequences of these proteins did not coincide with the endogenous GHF9 cellulases of the termite (Table 1). Notably, the amino acid sequence of HG 67-5 was identical to one of the *M. darwiniensis* GHF45 cellulases (GenBank accession number CAD39197), previously sequenced from protozoan cells of *M. darwiniensis* (9).

Identification of the SFM cellulase. The fractions SFM 68 and 76 (Fig. 1) were analyzed by SDS-PAGE. As shown in Fig. 3, SFM 68 contains several bands, while SFM 76 contains only two bands. Since only the major band of SFM 76 (at 50 kDa; named SFM 76-50K) contained an adequate amount of protein for N-terminal amino acid sequencing, this band was excised and analyzed using a protein sequencer. The resulting amino acid sequence was identical to sequences of GHF9 cellulases previously reported from the salivary glands (9, 27) and the midgut (23) of the termites (Table 1).

Comparison of hindgut and SFM cellulases. Our gel filtration analyses showed that the hindgut of *M. darwiniensis* contained at least three major cellulase components, which have different molecular sizes from the cellulases present in the salivary glands, foregut, and midgut of the termite. Based on comparisons between molecular weights and elution volumes of protein markers on the gel filtration chromatography column, approximate molecular weights (MW) of eluted proteins can be calculated by the following equation (3): $MW = 1633.2\ e^{-0.0428\ (\text{elution volume [ml]})}$. The molecular masses of the hindgut major peaks of cellulase activity are estimated to be 52.3 kDa, 57.9 kDa, and 71.1 kDa for the fractions HG 67, 65, and 61, respectively. The molecular masses of the major SFM peaks, which contain endogenous termite cellulases, are 49.6 kDa and 32.9 kDa for the fractions SFM 68 and 76, respectively. These data strongly suggest that most of the hindgut cellulases are different proteins at least in terms of size from those present in the salivary glands, foregut, and midgut.

Although the molecular weights of proteins eluted in the fraction HG 67 are close to those in the fraction SFM 68, our N-terminal amino acid sequencing indicated that fraction HG 67 does not contain GHF9 cellulases of the termite. On the contrary, the amino acid sequence of the cellulase detected from HG 67-5 was identical to amino acids 12 to 21 of a putative GHF45 protozoan cellulase (GenBank accession number CAD39197) from this termite. It also shows high similarity (80 to 90%) to other protozoan GHF45 cellulases (GenBank accession numbers CAD39198 to CAD39200). Assuming that amino acids 1 to 11 of the protozoan GHF45 cellulase (inferred from mRNA) represent a signal peptide that is removed, the putative molecular mass of the protozoan GHF45 cellulase (CAD39197) without this peptide is estimated to be 22.2 kDa. Since the estimated size of the cellulase that was eluted in the fraction HG 67 is slightly less than 50 kDa, our results suggest that the protozoan GHF45 cellulase was eluted as a dimeric form, although the form of this protein in vivo is unknown. On the other hand, the shift of molecular masses of SFM 76-50K (50 kDa) to a smaller size (32.9 kDa) during gel filtration can be explained by nonspecific (hydrophobic and/or ionic) interactions of this protein to the column materials, which usually delay elution (6, 10). The expected size of the termite-derived cellulase is 47 kDa.

Cellulose digestion in *M. darwiniensis*. The total amounts of cellulase activities present in SFM or the hindguts were inferred from the peak area of the elution profiles of cellulase activity during gel filtration (Fig. 1). Based on this, the present study supports the results of Veivers et al. (25), who demonstrated that the salivary glands and the hindgut, respectively, contribute 38% and 40% of total cellulase activity of *M. darwiniensis* (25). If all of the major peaks of cellulase activity in the hindgut extract originate from the symbiotic protozoa (as suggested by this study), it is clear that intestinal flagellates contribute significantly to cellulose digestion in *M. darwiniensis*.

On the other hand, Li et al. reported acquisition of the GHF9 cellulases by the hindgut protozoa and the presence of no significant activity of the protozoan GHF45 cellulases in *M. darwiniensis* (9). The present experiments revealed that the GHF9 cellulase was eluted in fraction SFM 76. The elution profile of the hindgut cellulase activity showed only a trace of activity around the corresponding position to SFM 76 (Fig. 1, HG 75 to 80). Thus, it is unlikely that GHF9 cellulases contribute significantly to cellulose digestion in the hindgut of *M.*

darwiniensis. One explanation for why Li et al. (9) found only GHF9 cellulases in the protozoan extracts is that their study was based on laboratory termites, whereas our study was based on wild termites. An alternative explanation is that the anion exchange MonoQ column preferentially adsorbed GHF9 cellulases rather than GHF45 cellulases. Indeed, MonoQ columns have previously been used to purify GHF9 cellulases from wood-feeding cockroaches (19, 20). During PAGE of a crude flagellate preparation (Fig. 3, lane 3, of Li et al. [9]), results were consistent with those of MonoQ-purified preparations. This may have been due to the fact that crude preparations were vortexed rather than homogenized, which may have led to the removal of protozoan cellulases during centrifugation.

The present study revealed that at least three cellulase components are involved in cellulose digestion in the hindgut of *M. darwiniensis* on the basis of the results of gel filtration chromatography, although we examined only one of these (HG 67-5) in detail. Further study is required on additional hindgut components. Although the actual origins and roles of each cellulase have yet to be clarified, it is obvious that the contribution of the protozoan cellulases to cellulose digestion in the hindgut of *M. darwiniensis* is not negligible. The important role of protozoan enzymes in cellulose digestion of lower termites has been demonstrated repeatedly throughout the history of termite research (11, 12, 13, 14, 15, 21, 26, 28, 30), and we conclude that *M. darwiniensis* is not an exception.

ACKNOWLEDGMENTS

Experiments performed at the National Institute of Agrobiological Sciences (enzyme purifications and amino acid sequencing) were supported by the Insect Technology Project of the Ministry of Agriculture, Forestry, and Fisheries of Japan (1 April 2003) to H.W. Sample collection and experiments done in Australia (species identification, dissection, and tissue preparation) were supported mainly by the Grant-in-Aid for Scientific Research (B) no. 17405025 from the Japan Society for the Promotion of Science to G.T.; additional support was from the Insect Technology Project to H.W., from an Australian Research Council Postdoctoral Fellowship to N.L., and from a Postdoctoral Fellowship by the 21st COE program of University of the Ryukyus to A.Y.

Export of extracts and dissected tissues of *M. darwiniensis* was conducted with the permission of the Australian government (no. WT2005-12842).

We thank H. Yamasaki of University of the Ryukyus for useful comments and kind assistance on the present study.

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